COMPLEX IMMUNO-GENE MEDICAL COMPOSITION FOR INHIBITING TUMOR CELLS

BACKGROUND OF THE INVENTION

1.Field of the Invention

[0001] The present invention relates to a medical composition for inhibiting tumor cell growth. In particular, it relates to a complex immuo-gene medical composition for inhibiting tumor cell growth, and a method for inhibiting tumor cell growth using the medical composition thereof.

2. The Prior Arts

[0002] Canine transmissible venereal tumor (CTVT) is naturally occurred, poorly differential tumor cells. The growth of the CTVT is similar to an allograft. CTVT in Canines are transmitted to each other, by mating, biting or contacting, by viable tumor cells through injured skin or mucus.

[0003] In experiment model, CTVT shows an expectable growth pattern. The growth phases in the CTVT model include Progressive phase (P phase), Stasis phase (S phase), and Regressive phase (R phase). CTVT expresses little major histocompatibility (MHC) molecules in P phase but large amount of transforming growth factor-β (TGF-β) in P phase and R phase TGF-β. is capable of inhibiting expression of MHC I and MHC II, and inhibiting activity of natural killer (NK) cells. Besides, 85 % of tumor infiltrating lymphocytes (TILs) of CTVT are lymphocytes being non-T or non-B cells, which are not able to express antigens characterized as T-cells and B-cells. From morphological observation, the aforementioned cells

contain big granules in their cytoplasm similar to those found in NK cells. It is presumed that the non-T or non-B cells shall be NK cells.

[0004] Major histocompatibility complex (MHC) class I antigens are 44 kDa glycoprotein expressed on the cell plasma membrane associated with β_2-microglobulin (β_2m), and it also called histocompatibility leukocyte antigens (HLA) in humans.

[0005] Tumor cells grow by escaping the monitoring of the host immune system through many different mechanisms. One of the mechanisms used by tumor cells is no or low expression of MHC class I antigens. For examples, Human tumor cells including primary breast carcinoma, advanced renal cell carcinoma, melanoma, prostate cancer, lung carcinoma, and other tumor source from colon, bladder, skin, endometrium have been found to express low MHC levels, and some cancer cells even express no MHC. In animals, low expression of MHC is also found in T lymphoma caused by Marek's disease virus in poultries and CTVT occurred in canines.

[0006] According to the "missing self" hypothesis, when tumor cells express low or no MHC, the activating receptors on the surface of the natural killer (NK) cells in host are activated, and the NK cells recognize and kill the target cells. However, many tumor cells secrete transforming growth factors to inhibit the cytotoxicity of NK cells. Therefore, the incapability of host immune system to function normally is one of the reasons for the tumor cells to grow rapidly without much of the constraint.

[0007] TGF-β is a 25 kDa homodimerric protein with very potent pleiotropic regulatory effects on the mammalian immune system. Addition of exogenous

TGF-β to culture of lymphocytes decreases proliferation of B cells, mature T cells, thymocytes, NK cells and lymphokine-activated killer (LAK) cells. Currently, It is known that many tumor cells including colorectal cancer, mammary tumor, thyreoglandular cancer, hepatocellular carcinoma (HCC) and Meth A tumor produce TGF-β . TGF-β is capable of helping growth of tumor by enhancing angiogenesis and cell adhesion. Moreover, the tumor cells evade the host immune surveillance by low MHC and low intercellular adhesion molecule-1(ICAM-1) expressions.

[0008] An antagonism or reduced secretion of TGF-β is a possible way to restore the normal function of host immune system and fight against tumor cells. Methods of immuno-therapy to inhibit TGF-β are presented, administration of TGF-β antibody by injection and gene therapy with antisense oligonucleotide are the other two major methods.

[0009] T lymphocytes cannot function to kill tumor cells that secret TGF-β or express no or low MHC. Accordingly, NK cells play important roles in against tumor cells expressing no or low MHC. NK cells differentiation-related cytokines are proved to be successful in removal of tumor cells. Those cytokines including γ- interferon (INF-γ), interleukin-2 (IL-2), IL-12, IL-15, IL-18 and IL-21. They are related to the functions of T lymphocytes, B lymphocytes, NK cells and other immune cells.

[0010] It is demonstrated that several cytokines are effective in against tumor cells in some in vitro and animal experiments. Those researches draw great attention to apply cytokines to cancer therapy. There are two kinds of cytokines that have been used against cancer cells. One kind is T-helper type 1 (Th1) cytokine, which stimulates reactions related to IL-2 and INF-γ production, and to the following cellular immunity, including IL-2, IL-12, IL-15, IL-18 and INF-γ. Another kind is

T-helper type 2 (Th2) cytokine, which stimulates reactions related to production of IL-4, IL-5 and IL-6 (these three cytokines stimulate B lymphocytes to grow and differentiate), and induces humoral immunity. However, results from those clinical researches using immuno-therapy of cytokines reveal that factors such as the way of administration, administrated amount, kinds of tumors, other additional cytokines or drugs, or potential side effect, and so on, are important in therapeutic effectivity and clinical application. Although immuno-gene-therapy is effective against tumor cells, there is limitation in practical application for the conventional methods. Usage of a combination of various cytokines according to the immune characteristics of tumor cells may be a more applicable method to fight against tumors.

SUMMARY OF THE INVENTION

[0011] A primary object of the present invention is to provide a complex immuno-gene medical composition for inhibiting the growth of tumor cells. The composition is capable of restoring the cytotocicity of NK cells by antagonizing TGF-β inhibitory effect on the host immune system.

[0012] Another object of the present invention is to provide a complex immuno-gene medical composition, which further activates the cytotocicity of NK cells in host immune system.

[0013] Some tumor cells express low MHC to escape the specific attack from host immune system (CD8+ T lymphocytes) in the growth period, but the low or no expression of MCH activates NK cells. In the meanwhile, tumor cells secret high level of TGF-β to inhibit differentiation and activity of NK cells. Moreover, TGF-β reduces the numbers and inhibits cytotoxicity of NK cells, lowers expression

of IFN-α and α chain of IL-2 receptor on cellular surface, and reduces secretion of INF-γ.

[0014] The present invention provides a complex immune-gene medical composition according to the aforementioned mechanism used by tumor cells to evade the host immune surveillance. The composition is capable of activating immune system by activating NK cells. The composition is the usage of a combination of a plurality of cytokines, that is, combined usage of the kinds of Th1 and Th2 cytokines. Th2 cytokines antagonize TGF-β inhibiting NK cells to disable the inhibition of immune system, and Th1 cytokines activate NK cells in host to enhance the ability fighting against tumor cells. By means of the complex immuno-gene medical composition, removal of tumor cells is expectable.

[0015] The aforementioned Th1 cytokines include IL-2, IL-12, IL15, IL-18 and INF-γ, and so on. And the aforementioned Th2 cytokines include IL-4, IL-5 and IL-6, and so on.

[0016] To demonstrate the inhibition effect of the composition according to the present invention to tumor cells, CTVT is used as a tested tumor model. Some reasons for choosing CTVT as a tested tumor model are described in the followings: (1) CTVT is a kind of tumor expressing low MHC; (2) CTVT produces a lot of TGF-β molecules; and (3) about 85% of tumor infiltrating lymphocytes (TIL) isolated from CTVT, expressing no CD3 and CD21, and is not T cells or B cells, are presumed as NK cells of canines.

[0017] Firstly, a plasmid containing human IL-6 coding sequence and another plasmid containing human IL-15 coding sequence are constructed with conventional

methods, respectively. Sequences of the constructed plasmids are confirmed. Expressed IL-6 and IL-15 is recovered after transfection *in vitro* and tested to evaluate the protein functionality.

[0018] Also, the effect for restoring cytotoxicity of NK cells *in vitro* is examined by using IL-6 and IL-15 together or each alone. *In vivo*, expression of IL-6 and IL-15, distribution of lymphocytes in spleen and cytotoxicity of NK cells are evaluated after introducing of constructed plasmid of IL-6 and IL-15 into BALA/c mice. Besides, C.B-17 SCID mouse is used as an animal model for testing the cytokines on the effect of NK cell function and subsequent activity of NK cells on preventing the growth of CTVT tumor cells, because the SCID mouse has immunologic deficiency in B and T lymphocytes but keeping functional NK cells. The effects of the present complex immuno-gene medical composition in activating NK cells and against CTVT are evaluated.

[0019] From the results of the above bioassays, it is demonstrated that the medical composition of the present invention is efficient in antagonizing TGF-β inhibitory effect on NK cells, restoring inhibited host immune system, activating NK cells in host, and enhancing ability of the host immune system in against tumor cells. The medical composition including the combination of the kinds of Th1 and Th2 cytokines is against tumors not only by antagonizing TGF-β produced by the tumor cells with low expression of MHC, but also by taking advantage of other immune responses. The present invention provides multiple strategies to inhibit growth of tumor cells.

[0020] Having been fully described the present invention, examples illustrating its practice are set forth below. These examples should not, however, be considered to

limit the scope of the invention, it is contemplated that modifications will readily occur to those skilled in the art, which modifications will be within the spirit of the invention and the scope of the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The related drawings in connection with the detailed description of the present invention to be made later are described briefly as follows, in which:

[0022] **FIG. 1** shows influence of IL-6 on cytotoxicity of NK cells. The E/T ratio is 13/1, there are triple samples in each treated group (N=3). X-axis represents the concentration of IL-6; Y-axis represents the specific lysis percentage (cytotoxicity) of YAC-1 cells.

[0023] **FIG. 2** shows comparison of effect of IL-15 and IL-2 to restore the ability of NK cellular cytotoxicity inhibited by TGF-β. The E/T ratio is 13/1, there are triple samples in each treated group (N=3). X-axis represents the groups treated; Y-axis represents the specific lysis percentage (cytotoxicity) of YAC-1 cells. NC is the control group without treatment of cytokines.

[0024] **FIG. 3** shows influence of combined usage of IL-6 and IL-15 on NK cellular activity inhibited by TGF-β. The E/T ratio is 13/1, there are triple samples in each treated group (N=3). X-axis represents the groups treated; Y-axis represents the specific lysis percentage (cytotoxicity) of YAC-1 cells. NC is the control group without treatment of cytokines.

[0025] **FIG. 4** shows distribution of splenocytes after delivery of IL-6 and IL-15 plasmids into BALB/c mice body with electroporation (N=3). A: Percentage of

CD3+T cells in the treated groups. B: Percentage of CD19+B cells in the treated groups. C: Percentage of NK1.1+NK cells in the treated groups.

[0026] **FIG. 5** shows NK cellular cytotoxicity of splenocytes after delivery of IL-6 and IL-15 plasmids into BALB/c mice body with electroporation (N=4).▲ represents the group treated with Mock vector; □ represents the group treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid; black ◊ represents the group treated with IL-6 and IL-15 plasmid together; ■ represents the group treated with IL-6 and IL-15 plasmid together and administration of anti-asialo GM1 antibody.

[0027] **FIG. 6** shows influence of complex immuno-gene therapy on tumor establishment of CTVT. ▲ represents the group treated with Mock vector; □ represents the group treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid; black ◊ represents the group treated with IL-6 and IL-15 plasmid together; ■ represents the group treated with IL-6 and IL-15 plasmid together and anti-asialo GM1 antibody administration; x represents the group treated with IL-6 and IL-15 plasmid together and administration of normal rabbit serum.

[0028] **FIG. 7** shows tumor growth curve of CTVT after therapy of complex immuno-gene adminstration. There are 6 mice in each treated group (N=6). ▲ represents the group treated with Mock vector; □ represents the group treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid; black ◊ represents the group treated with IL-6 and IL-15 plasmid together.

[0029] **FIG. 8** shows influence of complex immuno-gene therapy on survival rate of mice (N=5-6). ▲ represents the group treated with Mock vector; □ represents the group treated with IL-6 plasmid; ◊ represents the group treated with IL-15 plasmid;

black ◊ represents the group treated with IL-6 and IL-15 plasmid together; ■ represents the group treated with IL-6 and IL-15 plasmid together and administration of anti-asialo GM1 antibody.

[0030] **FIG. 9** shows influence of blocking of NK cellular function on the effect against CTVT tumor growth (N=6). + represents group treated with anti-asialo GM1 antibody; - represents the group being control experiment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0031] Because it is difficult to extract IL-6 gene from the body of canines, and there is very little IL-15 mRNA in the tissues of normal animal body, the sequence encoding human IL-6 (SEQ. ID. NO: 1) obtained from a IL-6 plasmid and a chimeric sequence encoding human IL-15 (IL-2 SP/IL-15 MP chimeric gene, SEQ. ID. NO: 4) are used in the present invention. The chimeric sequence contains an artificial sequence encoding the signal peptide of IL-2 (SEQ. ID. NO: 2) and a sequence encoding human IL-15 (SEQ. ID. NO: 3).

[0032] Commercial pcDNA3.1/V5-His-TOPO TA Expression Kit is applied to clone and construct the plasmids containing IL-6 gene and IL-2 SP/IL-15 MP chimeric gene, respectively. The constructed plasmids are transformed into E. coli cells (for example, from One shot® TOP10 competent E. coli) according to conventional methods. Also, PCR restriction enzyme cleavage, and DNA sequencing are employed to confirm the sequence. The plasmids with confirmed sequence are amplified, and purified with a Nucleobond AX plasmid purification kit (Macherey-Nagel, Durën, Germany).

[0033] Conventional MTS test is carried out to determine the activity of IL-6 in supernatant after transfection. The cell line of TF-1 (ATCC No. CRL-2003) which is dependent on IL-6 as a growth factor is employed to check the biological function of the IL-6 expressed by constructed IL-6 plasmid. The supernatant obtained after tranfection of the constructed IL-6 plasmid into Balb/3T3 cells (ATCC No. CCL-163) stimulates proliferation of TF-1 cells. And there is no stimulating activity in the supernatant obtained from a transfection of the pcDNA3.1/V5-His-TOPO vector into Balb/3T3 cells (ATCC No. CCL-163) or Balb/3T3 cells cultured alone. The result demonstrates that the constructed IL-6 plasmid expresses IL-6 protein with biological function.

[0034] The method evaluating the biological function of IL-15 expressed by constructed IL-15 is similar to the above method, but the cell line of TF-1 is replaced by HT-2 (ATCC No. CRL-2003) which is dependent on IL-15 as a factor for cell proliferation. The supernatant obtained from the culture of Balb/3T3 cells after transfection of constructed IL-15 plasmid is added into the culture of HT-2 cells. The supernatant obtained after tranfection of the constructed IL-6 plasmid into Balb/3T3 cells stimulates proliferation of HT-2 cells. And there is no stimulating activity in the supernatant obtained after transfection of the pcDNA3.1/V5-His-TOPO vector into Balb/3T3 cells or Balb/3T3 cells cultured alone. The result demonstrates that the constructed IL-15 plasmid expresses IL-15 protein with biological function.

[0035] Moreover, the effect of IL-6 and IL-15 on antagonizing TGF-β inhibitory activity of NK cells are examined *in vitro*. Comparing to the usage of IL-6 or IL-15 alone, there is higher cytotocicity to YAC-1 mice lymphoma cells in usage of IL-6 and IL-15 together. The result reveals that the usage of IL-6 alone recovers the function of

NK cells inhibited by TGF-β, but IL-6 alone is not capable of activating NK cells and thus, the effect of cytotoxicity is limited. In the same way, the usage of IL-15 alone can activate NK cells, but it cannot remove TGF-β. Therefore, the activity of NK cells is inhibited and cannot be restored completely. The usage of IL-6 and IL-15 together provides IL-6 to antagonize TGF-β inhibiting immunity of NK cells, furthermore, IL-15 to activate NK cells. The cytotoxicity of NK cells is elevated effectively through the combined usage of IL-6 and IL-15.

[0036] For evidencing the effect of the combined usage of IL-6 and IL-15 plasmids, the constructed IL-6 and IL-15 plasmids are muscle electropolated alone or together into the body of BALB/c mice *in vivo*, and the splenocytes of the BALB/c mice are examined. The method of muscle electroporation is widely used in non-viral vector delivery in gene therapy recently. Comparing to viral vector, the non-viral vectors are preferred because the advantages including high safety, low immune response, good efficiency in plasmid delivery, good protein expression, and near body surface to easily operate. The results from ELISA show expressions of IL-6 and IL-15 in host mice are successful.

[0037] Fourteen days after muscle electroporation, the numbers and cytotoxicity of NK cells in the treated mice spleens are elevated more obviously in usage of IL-6 and IL-15 plasmid together than the usage of IL-6 plasmid or IL-15 plasmid alone. Moreover, the numbers and cytotoxicity of NK cells in usage of IL-15 plasmid alone at E/T ratio of 50/1 and 12.5/1 are lower than in usage of IL-6 and IL-15 plasmid together, but higher than in usage of IL-6 plasmid alone and in usage of vector. It reveals that IL-15 itself is capable of enhancing activating of NK cells.

[0038] C.B-17 SCID mouse is an artificial breeding strain with

immunodeficiency. The mouse has no mature T cells and B cells with normal functionality, but it has normal myeloid cells, antigen-presenting cells (APCs) and NK cells. The mice are inoculated with CTVT for xenotransplantation. And muscle electroporation is carried out to deliver IL-6 and IL-15 plasmids into the mice body. First, influences of IL-6 and IL-15 on tumor establishment are evaluated. Secondly, the inhibiting effects of the two cytokines against established tumors are also evaluated. The experimental results demonstrate that combined usage of IL-6 and IL-15 plasmid is most effective in inhibiting the establishment of the tumor, and IL-15 plasmid alone is little effective to inhibit tumor establishment. No obvious influence is observed in the usage of IL-6 plasmid alone. In addition, only combined usage of IL-6 and IL-15 plasmid is effective to reduce growth rate of the established CTVT. IL-6 plasmid or IL-15 plasmid alone cannot suppress the growth of established tumor. In another experiment, anti-asialo GM-1 antibodies, an antibody that blocks the function of NK cells, is intraperitoneally injected into the SCID mice that carry the tumor. Then, the combined composition of IL-6 and IL-15 plasmid is delivery into the mice. This combined composition cannot suppress the growth of CTVT. The result reveals that NK cell plays an important role in such method of complex immuno-gene therapy.

Example 1: Preparation and synthesis of IL-6 and IL-15 genes

[0039] According to the mRNA sequence encoding human IL-6, Accession No. NM_000600 from Genbank, NCBI pubmed (http: // www. ncbi. nlm. nih. gov) , IL-6 gene containing 636 bases (the sequence from the base 63 to 698 is as SEQ ID NO: 1) in whole length is amplified and obtained with conventional methods.

[0040] The sequence encoding human IL-2 signal peptide (IL-2SP, 60 base, base

461 to 107, SEQ ID NO: 2) is linked to the sequence encoding human IL-15 mature peptide (IL-15MP, 342 base, base 461 to 802, SEQ ID NO: 2) to form a IL-2 SP/IL-15 MP chimeric gene (SEQ ID NO: 4) according to the design presented by Kazuhiro et al. (2001). The design overcomes the disadvantage that it is via multiple regulators in transcription and translation and leads to low production of IL-15 protein. In amplification, the sense strand of chimeric gene sequence is synthesized with 13 primers, which primers are from the chimeric gene sequence, total length of 402 bases, fractionated by every 30 bases from the direction of 5'end. The antisense strand is synthesized with another 13 primers as the linked bridges, which sequences are complemented to the last and the next sense strain with 15 bases after the 15th base from the starting base.

[0041] The human IL-6 coding sequence is obtained from a PCR product amplified and purified from a IL-6 plasmid (from National Taiwan University College of Medicine, Taipei, Taiwan) which is created by inserting a human IL-6 gene into pcDNA3. The human IL-2SP/IL-15MP chimeric gene is obtained from a PCR product amplified with aforementioned 26 primers and purified. The sequences of primers in antisense strand are complemented to the last and next sense strain with 15 bases. The primer anneals to the sequence complementary to its own sequence in the reaction with DNA polymerase to form a template. The product of IL-2SP/IL-15MP chimeric gene is obtained from PCR reaction.

Example 2: Cytotoxicity evaluation of NK cells in mouse spleen *in vitro*

[0042] The BALB/c mice, aged 6 to 8 weeks, are sacrificed and the spleens are taken. The spleens are homogenized in RPMI-1640 medium to obtain a single cell suspension. The cell suspension is centrifuged at 1,500 rpm and 4°C

for 10 minutes. After discarding the supernatant, the cell pellets are added into 5 ml of 1 X ACK lysis buffer (10 X ACK lysis buffer contains 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA) to lyse red blood cells for 5 minutes at room temperature. The cell lysate is added into 10 ml of RPMI-1640 medium, centrifuged at 1,500 rpm and 4 °C for 10 minutes. After discarding the supernatant, the cells are rinsed with RPMI-1640 medium once. Count the cell numbers and dilute the cells in LAK medium which is RPMI-10 medium containing 50 μM 2-mercaptoethanol and IL-2, the ratio of IL-2 to cells is 500 U to 1 x 10^6 viable cells. The Cell solution is cultured with 2 x 10^6 cells/2 ml per well in culture 24 well-plate at 37 °C for 4 to 6 days in 5% CO_2 incubator. After 3 days from culturing, IL-2 is added into the culture in a ratio of 500 U to 1 x 10^6 viable cells again. To evaluate the influence of IL-6, IL-15, or TGF-β onto cytotoxicity of NK cells of mice splenocytes, the method for culturing of spleen cells is in accordance with the above description but the lymphokine is replaced by the cytokine being interested.

[0043] YAC-1 lymphoma cells (ATCC No. TIB-160) from mice are rinsed with PRMI-1640 medium twice with centrifugation at 1,500 rpm and 4°C for 10 minutes, and counted with Trypan Blue Eexclusion Test. The cells are suspended in PRMI-1640 medium with 5 x 10^5 viable cells/ml, and the cell numbers are determined with effector cells. The fluorescence dye of 3,3'-dioctadecyloxacarbocyanine (DIOC 18, Sigma, MO, USA) is added into the cell suspension in a ratio of 10 μl to 5 x 10^5 viable cells/ml, and incubated at 37 °C for 16 hours in 5% CO_2 incubator for reaction. After rinsing twice with centrifugation at 1,500 rpm and 4°C for 10 minutes, the cells are suspended into RPMI-10 medium.

[0044] The splenocytes and YAC-1 cells according to the mentioned treatments are mixed with various Effector/ Target ratio (E/T ratio), 50/1, 25/1, 12.5/1, 6.25/1, 3.125/1 and added into the wells with round bottom of a 96 well-plate (200 μl at most per well). The mixtures are centrifuged at 1,100 rpm and 4 °C for 5 minutes, and then incubated at 37 °C for 4 hours in 5% CO_2 incubator in dark. After incubation, the cells are harvested and mixed with propidium iodine (PI) (2500μm/ ml) which volume is 1/100 of cell solution. The cells are analyzed with flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, NJ, USA) and CellQuest software.

[0045] The results for IL-6 stimulating splenocytes of BALB/c mice show that the specific lysis percentage of YAC-1 lymphoma cells of mice dose not increase with increased IL-6 dosage (**FIG. 1**). After 6 days for activating splenocytes with cytokines, the specific lysis percentage in the group treated with 400U of IL-15 is similar to the group treated with 2000 U of IL-2. The cytotoxicity of NK cells is completely inhibited when the cells are cultured with both IL-2 and TGF-β, but partially inhibited when cultured with IL-15 and TGF-β together. The results reveal that IL-15 itself is superior to IL-2 for activating NK cells, and restoring the NK cell cytotoxicity inhibited by TGF-β (**FIG.2**). Mice splenocyte culture with IL-6 and TGF-β together, with IL-15 and TGF-β or TGF-β alone show that IL-6 is not capable of promoting cytotoxicity of NK cells, but IL-15 slowly promotes the NK cellular cytotoxicity inhibited by TGF-β. The most significant promoting effect is found when IL-6 and IL-15 are used together. (**FIG. 3**).

Example 3: Influence of IL-6 and IL-15 gene delivery with electroporation on cytotoxicity of NK cells from BALB/c mice

[0046] The BALB/c mice are divided into four groups for different treatments: (1)

treatment with 100 μg of pcDNA3.1/V5/His/TOPO (Mock) vector; (2) treatment with 100 μg of IL-6 plasmid; (3) treatment with 100 μg of IL-15 plasmid; and (4) treatment with 100 μg of IL-6 plasmid and 100 μg of IL-15 plasmid together. There are 6 mice in each group. The solutions for treatment such as vector, IL-6 plasmid and IL-15 plasmid solution are prepared as the concentration of 1 mg/ml with saline before electroporation.

[0047] BALA/c mice are anesthetized and injected 50 μl of vector or plasmid solution into both sides of muscles, respectively. Standing for 2 minutes to diffuse the injected solution into muscles, the mice are electroporated with electroporator (Electro Square Porator, BTX ECM 830), and the electroporation is carried out at 0.5 cm of inserting depth, 100 volts for 10 times, and 50 ms each time. After electroporation treatments, the blood samples are taken on day 0, 3, 8, 12, 14, 15, 20, 25, 27 and 30. The blood samples are centrifuged to recover the serums and stored at -20 °C.

[0048] To examine the protein expression of IL-6 and IL-15 plasmid *in vivo*, the commercial ELISA kit (IL-6: Endogen, MA, USA; IL-15: Biosource, CA, USA) is applied to serum samples to assay the concentration of IL-6 and IL-15 with a conventional procedure.

[0049] The results demonstrate both two plasmids express proteins in the mice bodies. IL-6 is detectable in serum samples in all groups, but the concentration and expressed time are not in accordance. IL-15 is detectable only in the group (3) and (4).

[0050] On day 14 after electroporation, 4 mice are sacrificed for each group to

collect the spleen cells, the lymphocytes subpopulations (T, B cells and NK cells) and cytotoxcity of NK cells are evaluated.

[0051] Spleens taken from the electroporated mice, are homogenized to obtain splenocytes. A 100 μl splenocytes suspensions of 1 x 10^7 viable cells/ ml are incubateded with various monoclonal antibodies including rat-anti-mouse CD3-FITC antibody (Serotec, Oxford, UK), rat-anti-mouse CD19-FITC antibody (Serotec, Oxford, UK), rat-anti-mouse NK1.1-FITC antibody (PharMingen), and other isotypes. A 100 μl of splenocyte solution is added into 1 μg of antibody, reacted at 4 °C for 45 minutes, and then rinsed with 1 X PBS solution twice. To assess cell viability, 500 μg/ml of propidium iodine is added into the cell solution. The cell subpopulations are analyzed with flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, NJ, USA).

[0052] From the results, there are no obvious differences in percentage of T cells and B cells among the four experimental groups (**FIG. 4 A and B**), and percentage of NK cells is significantly elevated in the treatment of group (4). In addition, group (4) shows superior NK cellular cytotoxicity to the other groups in whatever E/T ratios. Group (3) exhibits higher cytotoxicity significantly when E/T ratio is 50/1 or 12.5/1, but there is no significant difference when E/T ratio is 3.125/1. There is no significant difference between groups (1) and (2) in any E/T ratios (**FIG. 5**).

Example 4 : Effects of combined usage of IL-6 and IL-15 plasmid in inhibiting CTVT in C.B-17 SCID mice

[0053] CTVT is surgically excised from canine inoculated artificially, homogenized and passed through a two layer stainless mesh (No. 25) to obtain single

cell suspension. CTVT cells are isolated with 42% of Percoll (Amershampharmacia biotech, NJ, USA). Vital stain (Trypan Bblue Exclusion Test) is applied to determine viability of the tumor cells. C.B-17 SCID mice are subcutaneously inoculated with 1 x 10^8 viable CTVT cells with 18 G syringe on each side of the back. The size of the tumors is measured twice a week after inoculation of CTVT. The tumor size is determined according to the following formula:

$V = \pi \times L \times W \times H / 4$

wherein, V is volume of tumor (cm^3), L is length of tumor (cm), W is width of tumor (cm) and H is height of tumor (cm).

[0054] Electroporation is performed on day 7 post CTVT inoculation (tumor is not established yet) to observe the influence of IL-6 and IL-15 on CTVT establishment. It shows that the tumors reach an observable size (diameter is about 2-3 mm) after 14 days from CTVT inoculation. In the group of combined usage of IL-6 and IL-15 plasmid, the tumors are obviously smaller than those found in other groups within the period of observation. Group treated with IL-15 plasmid alone shows a smaller tumor size than group treated with IL-6 plasmid alone and treated with vector, but there is no significant difference among those treatments statistically ($p>0.05$). Tumor growth rate in the group treated with IL-6 plasmid alone is similar to the group treated with vector, and there is also no significant difference between the two groups.

[0055] Anti-asialo GM-1 antibody is dissolved in 1 ml of solution suitable for injection. Each mouse is intraperitoneal injected with 30 μl of the anti-asialo GM-1 antibody twice a week to block the function of NK cells, and then electroporated with IL-6 plasmid and IL-15 plasmid. The result reveals the growth rate of tumor is

dramatically increased, and the tumor size is bigger than the groups with other treatments.

[0056] Besides, C.B-17 SCID mice inoculated with CTVT cells are electroporated when the tumor reaches 5 mm. IL-6 and IL-15 together effectively delay the growth rate of established tumors. Three tumors (sample size is 6, and each mouse is inoculated two side) disappear and do not grow again. In the period of observation, the average tumor size of the combined treatment (IL-6 plus IL-15) is significant smaller than groups treated with vector, IL-6 plasmid or IL-15 plasmid alone. IL-6 plasmid or IL-15 plasmid alone is not effective in inhibiting the growth of the tumors, and the sizes of tumors are similar to the group treated with vector (**FIG. 7**).

[0057] Furthurmore, in order to realize the relationship between tumor inhibition effect shown by combined usage of IL-6 and IL-15 plasmid, and NK cells, 4 C.B-17 SCID mice are injected peritoneally with anti-asialo GM-1 antibody to block the function of NK cells. The results show that administration of IL-6 and IL-15 plasmid in the same level mentioned above is not able to suppress growth of tumors after injection of anti-asialo GM-1 antibody, and the survival rate is increased obviously (**FIG. 8** and **FIG. 9**). Thus, NK cells are the major cells to inhibit the growth of the tumor. IL-6 and IL-15 are effective cytokines to promote host NK activity in inhibiting the establishment of a tumor and in against an established tumor.